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ORIGINAL ARTICLE

Activating Cannabinoid Receptor 2 Alleviates Pathogenesis of Experimental Autoimmune Encephalomyelitis Via Activation of Autophagy and Inhibiting NLRP3 Inflammasome

Bo-Zong Shao,[1] Wei Wei,[2] Ping Ke,[1] Zhe-Qi Xu,[1] Jv-Xiang Zhou[1] & Chong Liu[1]

1 Department of Pharmacology, Second Military Medical University, Shanghai, China
2 State Key Laboratory Breeding Base for Zhejiang Sustainable Plant Pest and Disease Control, Zhejiang Province Key Laboratory for Food Safety, Institute of Quality and Standard for Agro-products, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

Keywords
Autophagy; Cannabinoid receptor 2; EAE; NLRP3 inflammasome.

Correspondence
C. Liu, 325 Guohe Road, Shanghai 200433, China.
Tel.: 0086-21-8187-1280-802;
Fax: 0086-21-6549-3951;
E-mail: wanlc2004@aliyun.com
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The first two authors contributed equally to this work.

SUMMARY

Aims: Activation of cannabinoid receptor 2 (CB2R) has been reported to ameliorate the pathogenesis of experimental autoimmune encephalomyelitis (EAE). In this study, we examined whether autophagy is involved in the beneficial effect of CB2R on EAE and explored the mechanism with a focus on inflammasome activation. **Methods:** EAE severity was analyzed with clinical score and histological score stained by hematoxylin and eosin or luxol fast blue in spinal cord. Immunoblot analysis was conducted to detect proteins of NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome-related caspase-1 (Casp-1) and the maturation of interleukin (IL)-1β as well as autophagy-related light chain 3 (LC3), and Beciln 1 both *in vivo* and *in vitro*. Reverse transcription and real-time PCR were used to detect mRNA of NLRP3, IL-1β and Casp-1. Autophagy-related gene 5 (ATG5)-specific siRNA was transiently transfected in BV2 microglia, and immunofluorescence staining was carried out to detect the expression of NLRP3, caspase recruitment domain (ASC), and pro-caspase-1. **Results:** The current data indicated that deleting CB2R decreased the expression of LC3-II/LC3-I ratio, Beclin 1 and increased caspase-1 activation and IL-1β production in the spinal cord of EAE mice, whereas activation of CB2R with a specific agonist HU-308 induced inverse effects. Further study indicated that HU-308 could promote autophagy and inhibit expression and activation of NLRP3 inflammasome in BV2 microglia. Blocking autophagy by ATG5-specific siRNA dismissed the effort of CB2R in mediating NLRP3 inflammasome *in vitro*. **Conclusion:** Collectively, our results demonstrated for the first time that CB2R plays a protective role in EAE through promoting autophagy and inhibiting NLRP3 inflammasome activation.

Introduction

Autophagy, initially viewed as a conserved bulk degradation mechanism, has emerged as a central player in both innate and adaptive immunity [1,2]. The important link between autophagy and immunity could be through playing a negative role with respect to inflammasome activation, which is responsible for the activity of caspase-1 (Casp-1) and the maturation of interleukin (IL)-1β and IL-18 [3]. Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease that is characterized by immune-mediated demyelination and neurodegeneration of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is a well-studied animal model of MS. Recent studies suggest that T helper 17 cells (Th17) are the major pathogenic T cells that mediate the pathogenesis of EAE [4]. IL-1 signaling is critical in the regulation of Th17 differentiation and finally deteriorates the pathogenesis of EAE [5].

A large number of studies suggest an important role for the cannabinoid system in the developments of MS and EAE. Both cannabinoid receptor 1 (CB1R)- and CB2R-knockout mice are more susceptible to the development of severe EAE [6,7], and the activation of these receptors improves clinical symptoms not only in rodent models of EAE [8], but also in humans with multiple sclerosis [9]. The role of CB2R, however, is directly involved with innate immune system because CB2R is primarily expressed in immune cells [10,11], which are intimately related to the inflammatory response in MS. Activation of CB2R ameliorate EAE through suppressing key components of the inflammatory process [12] and reducing Th17 differentiation, immune cell accumulation, and microglia activation in the CNS. Recent studies showed that cannabinoid induces autophagy in pancreatic cancer cells or human glioma cells [13,14], but whether activation of CB2R regulates autophagy in EAE remains unclear.

Here, we demonstrate that activation of CB2R could promote autophagy and inhibit inflammasome activation both in the spinal

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cord of EAE and in BV2 microglia. Blocking autophagy dismissed the effort of CB2R in mediating NLRP3 inflammasome activation *in vitro*. Our results demonstrated for the first time that CB2R plays protective roles in EAE through inducing autophagy and inhibiting NLRP3 inflammasome activation.

Materials and methods

Animal Care and Use

C57BL/6 mice were purchased from Shanghai Super-B&K Laboratory Animal Corp. Ltd. (Shanghai, China). CB2R-KO mice on a C57BL/6 background were purchased from Bar Harbor, MA (B6.129P2-Cnr2^{tm1Dgen}/J, Stock Number: 005786). All mice were maintained in pathogen-free condition with standard laboratory chow and water *ad libitum*. All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of Second Military Medical University.

EAE Induction and Assessment

EAE was induced in female mice at 8–9 weeks as previously reported [15,16]. In brief, mice were subcutaneously immunized with 200 μg MOG_{35-55} in Complete Freund's adjuvant (Sigma-Aldrich, St.Louis, MO, USA) containing heat-killed *Mycobacterium tuberculosis* (H37RA strain; 5 mg/mL; BD Diagnostics, Franklin Lakes, NJ, USA). Pertussis toxin (200 ng/mouse; Calbiochem, Billerica, MA, USA) was injected via i.p. on days 0 and 2. Mice were assessed daily for clinical signs by researchers blinded to experimental conditions and were assigned scores as follows: 0, no clinical signs; 1, paralyzed tail; 2, paresis; 3, paraplegia; 4, paraplegia with forelimb weakness or paralysis; and 5, moribund or death. For drug treatment, HU-308 dissolved in saline was injected via i.p. once daily from day 3 till the end of the study. Saline was given as vehicle control (100 μL/mouse).

Histopathological Analysis

The mice were anesthetized and perfused with PBS (pH 7.4) followed by 4% (w/v) paraformaldehyde. Spinal cord samples were then fixed in 4% (w/v) paraformaldehyde overnight. Paraffin-embedded sections were stained with hematoxylin and eosin or luxol fast blue to analyze inflammation or demyelination, respectively.

Culture and Treatment of BV2 Microglia

Murine BV2 microglia were cultured with Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% (vol/vol) fetal bovine serum (Gibco) at 37°C in a humidified incubator with 5% CO_2. After priming with 100 ng/mL LPS for 12 h and stimulating with ATP (1 mM), treated with vehicle or HU-308 (10 μM) for 10 min in advance, the cells were collected, and the mRNA level of NLRP3, IL-1β and Casp-1 were measured using real-time PCR.

Reverse Transcription and Real-Time PCR

Total RNA was extracted from BV2 microglia using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was subjected to reverse transcription with random hexamer primer and M-MLV reverse transcriptase (Progema, Madison, WI, USA). Real-time PCR was conducted in the LightCycler quantitative PCR apparatus (Stratagene, Santa Clara, CA, USA) using the SYBR Green JumpStart™ Taq ReadyMix™ kit (Sigma). Expression value was normalized to β-actin in the same sample and then normalized to the control. The sequences of the primer pairs are listed in the supplementary table 1.

Immunoblot Analysis

Spinal cords from EAE mice or BV2 microglia were washed once with PBS and lysed in lysis buffer by sonication for 30 s on ice. Protein concentration was determined by the BCA method (Thermo Scientific, Pittsburgh, PA, USA). The samples were loaded into 12% Tris/Gly gels, subjected to SDS-PAGE, and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). Western blotting was performed using the rabbit anti-LC3 polyclonal antibody (Novus Biologicals, Littleton, CO, USA), anti-Beclin 1 monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA), anti-Casp-1 monoclonal antibody (Abcam, Cambridge, MA, USA), goat anti-IL-1β monoclonal antibody (R&D, Minneapolis, MN, USA), and corresponding HRP-conjugated secondary antibody (Promega). Then, the image was captured and analyzed by the Odyssey infrared imaging system (Li-Cor Bioscience, Lincolin, NE, USA). All immunoblotting experiments were repeated for at least three times.

Transient Transfection and siRNA

ATG5-specific siRNAs and the control siRNA were purchased from GenePharma (Shanghai, China). The forward siRNA sequences targeting ATG5 were as follows: sense, 5′-CUCUCUAUCAGGAUGAGAUTT-3′ and antisense, 5′- AUCUCA UCCUGAUAGA.

GAGTT-3′. BV2 microglia were plated at a density of 2×10^5 on a confocal dish and cultured up to 90% confluence and then were transfected with control siRNA or ATG5 siRNA as described before [17,18]. After transfection, the BV2 microglia were incubated with HU-308 and then stimulated with LPS and ATP.

Immunofluorescence Staining and Fluorescence Microscopy

BV2 microglia were cultured in eight-well chambers and then pretreated for 10 min with HU-308 or vehicle before stimulated with LPS (100 ng/mL) and ATP (1 mM). Cells were fixed in 200 μL of 4% paraformaldehyde for 15 min, washed with phosphate-buffered saline (PBS) before blocking with 1% bovine serum albumin in PBS, and incubated with primary antibodies overnight at 4°C. The primary antibodies and concentrations used were as follows: goat anti-NLRP3 (1:100, Abcam) with rabbit anti-ASC

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(1:100, Santa Cruz Biotechnology, Dallas, TX, USA), or goat anti-NLRP3 with rabbit anti-Casp-1, (1:100, Santa Cruz Biotechnology, Delaware Ave Santa Cruz, CA, USA). Double immunofluorescent staining was completed by Alexa-488 or Alexa-647-labeled secondary antibody (1:500, Invitrogen) incubation for 1 h at room temperature. After being washed, slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and colocalization was observed using a confocal laser scanning microscope (Fluoview FV1000; Olympus, Tokyo, Japan). As described in our previous studies, Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) was employed to analyze colocalization, expressed as the Pearson correlation coefficient [19,20]. In this case, the experiments were performed in a double-blind manner.

Statistical Analysis

Data are presented as means ± SEM. The statistical significance of the EAE clinical scores between treatments was analyzed with a two-way ANOVA test. Other analyses were assessed by Student's *t*-test. P values <0.05 were considered statistically significant.

Results

Activating of CB2R Ameliorates Clinical Symptoms and CNS Infiltration in EAE

At a low dose of 0.3 mg/kg, HU-308 produced no effect on clinical score of EAE. However, 1 and 3 mg/kg HU-308 significantly reduced the peak severity and cumulative clinical score of EAE (Figure 1A). Histological examination of the spinal cords was performed at day 17 postimmunization (PI). Compared to vehicle control, HU-308 (1 mg/kg) caused a dramatic reduction of leukocyte infiltration in spinal cord (Figure 1B and D). Luxol fast blue staining also revealed less extensive demyelination in HU-308-treated mice than in controls (Figure 1C and E). These data indicate that activating CB2R signaling significantly alleviates EAE severity accompanied by reduced CNS inflammation and demyelination.

CB2R Mediates Inflammasome Activation in EAE

Inflammasomes are multiprotein complexes and serve as platforms for the activation of the Casp-1, which leads to the processing and secretion of the main proinflammatory cytokine such as IL-1β. In this study, CB2R-deficiency significantly increased both Casp-1 activation and IL-1β production in spinal cord of EAE mice (Figure 2A), while activation of CB2R with HU-308 induced inverse effects (Figure 2B). NLRP3 inflammasome is the most characterized inflammasome, and a number of studies suggested the involvement of NLRP3 inflammasome in the development of MS or EAE [21]. We also found that CB2R-deficiency significantly increased the NLRP3 mRNA expression (Figure 2C), whereas activation of CB2R with HU-308 decreased it (Figure 2D). These combined data suggest that CB2R negatively mediates NLRP3 mRNA expression and NLRP3 inflammasome activation both in brain and in spinal cord of EAE mice.

CB2R Increases Autophagy in EAE

Activation of autophagy leads to inflammasome destruction and limits IL-1β production [22]. Our data indicated that activation of CB2R inhibited NLRP3 inflammasome activation, so we speculated that autophagy might play a role CB2R-mediated NLRP3 inhibition in EAE. As LC3-II/LC3-I ratio and the expression of Beclin 1 have been established as useful sign for autophagy, we detected LC3-II/LC3-I ratio and Beclin by immunoblotting in spinal cord of EAE mice. CB2R-deficiency reduced the expression of LC3-II/LC3-I ratio and Beclin 1 (Figure 3A), whereas HU-308 increased it (Figure 3B). Taken together, these data indicate that activating CB2R induces autophagy in CNS of EAE, whereas blocking CB2R reduces it.

Autophagy Regulates CB2R-Mediated NLRP3 Inhibition in BV2 Microglia

CB2R was mainly expressed in Microglia in CNS. Microglia is the major antigen-presenting cells (APCs) in CNS which produces potent proinflammatory molecules including IL-1, IL-6, and tumor necrosis factor α (TNF-α) upon maturation [23]. Here, we observed the effect of activation of CB2R on autophagy and NLRP3 inflammosome in BV2 microglia. LPS/ATP stimulation significantly increased the expression of LC3-II/LC3-I ratio, Beclin 1, and NLRP3 inflammasome activation (Figure 4A–C). Treated with HU-308 significantly decreased the expression of autophagic protein and NLRP3 activation (Figure 4D–F), which are consistent with the data from *in vivo*.

The NLRP3 inflammasome is a major intracellular multiprotein complex consisting of three kinds of proteins, namely NLRP3, caspase recruitment domain (ASC), and pro-caspase-1 [24]. To determine whether inhibitory effect of CB2R on NLRP3 inflammosome is mediated by autophagy, we evaluated the influence of autophagy-related gene 5 (ATG5) siRNA on inhibitory effect of NLRP3 inflammasome formation mediated by CB2R activation using confocal microscopy analysis. In the control siRNA group, LPS-/ATP-induced colocalization (yellow spots) of inflammasome molecules (NLRP3 (green) vs. ASC or caspase-1 (red)) in BV2 microglia compared with control cells. Pretreatment with HU-308 significantly inhibited LPS/ATP-induced colocalization of NLRP3 with ASC or caspase-1, suggesting activation of CB2R inhibited the formation of NLRP3 inflammasomes. In the ATG5 siRNA group, we observed that knockdown of ATG5 attenuated the inhibitory effects of HU-308 on formation of NLRP3 inflammasomes. Collectively, these data suggested that the induction of autophagy mediates at least partly protective effect of HU-308 on NLRP3 inflammasomes formation (Figure 5).

Discussion

Several works have been reported so far to demonstrate the positive effects and dramatic significance of CB2R in attenuating MS or EAE [25], yet the specific molecular mechanism have not been described clearly. This is the first report showing that CB2R is protective in EAE through the activation of autophagy and inhibition of NLRP3 inflammasome in mouse microglia. In our study, activating CB2R with HU-308 significantly alleviated clinical

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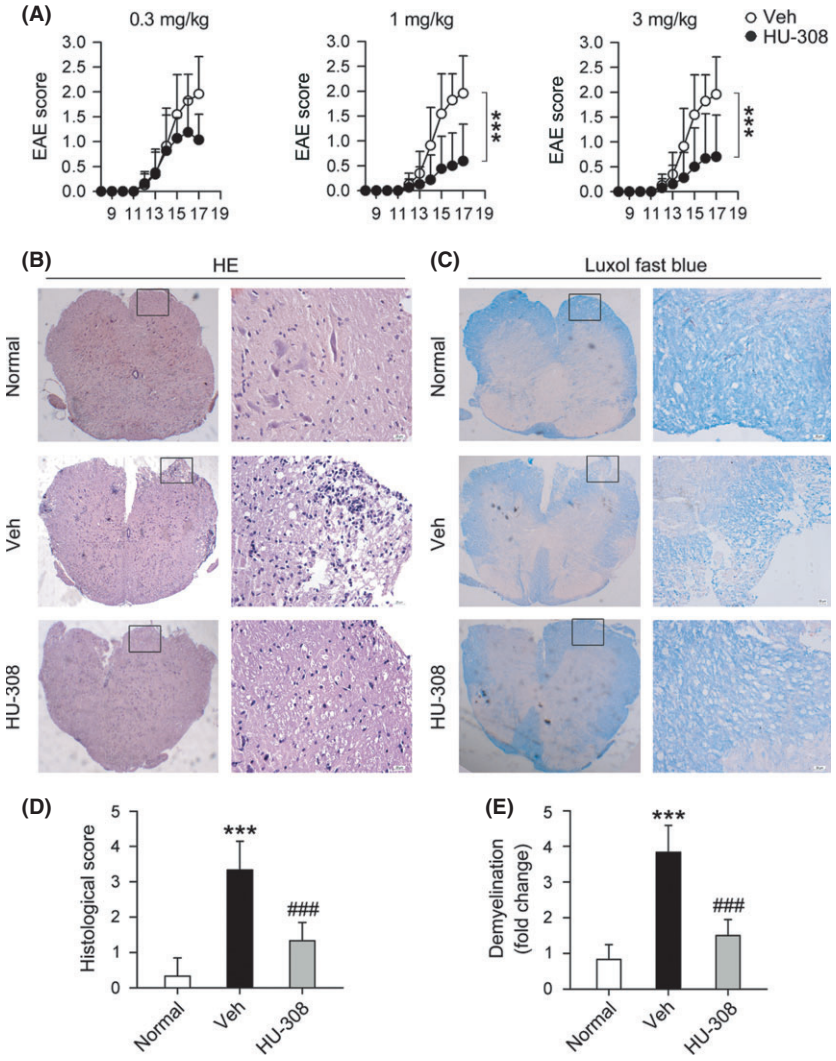

Figure 1 CB2R activation alleviates clinical symptoms *and CNS infiltration* in EAE mice. EAE mice were treated with HU-308 (0.3, 1 and 3 mg/kg/day, i.p.) or vehicle (Veh) from days 3 postimmunization (PI) and were maintained on drug for the duration of the study. (**A**) Clinical signs were assessed daily by researchers as described in *Methods*. 1 and 3 mg/kg HU-308 significantly reduced the peak severity and cumulative clinical score of EAE (n = 16 per group). $^{###}P < 0.001$ *vs* Veh. (**B**) H&E staining and (**C**) Luxol fast blue staining of paraffin sections of spinal cords isolated from normal, vehicle, or HU-308 (1 mg/kg)-treated EAE mice on day 17. (**D**–**E**) Quantification of CNS infiltrates and the amount of demyelination presented in **B** and **C**. (n = 6 per group). $^{***}P < 0.001$ versus normal, $^{###}P < 0.001$ versus Veh.

symptoms and CNS inflammatory infiltration, exhibiting less peak severity, leukocyte infiltration, and extensive demyelination in EAE mouse. Consistently, activation of CB2R with HU-308 decreased inflammasome expression and initiation, while CB2R knockout increased them in mouse EAE model. Compared with the WT mice, CB2R-KO mice showed lower expression of autophagy-related proteins, and activating CB2R induced inverse effect. In BV2 microglia, levels of autophagy-related proteins were increased, in combination of the upregulation of NLRP3, as well as infammasome-related IL-1β and Casp-1 in expression under the stimulation with LPS/ATP. However, activating CB2R further increased autophagy, but inhibited NLRP3, IL-1β and Casp-1 in expression under LPS/ATP stimulation. Furthermore, inhibition of autophagy enhanced NLRP3 inflammasome in BV2 microglia. Taken together, these data showed that both activation of autophagy and inhibiting NLRP3 inflammasome were involved in the protective effect of CB2R on EAE.

MS is a demyelinating disorder which leads to inflammation, demyelination, and axonal injury [26]. Chronic inflammation and neurodegeneration are currently considered as two main features of MS pathology [27]. Cannabinoid and some of its derivatives have been largely proved to produce positive and protective roles in MS or EAE [28], mainly through the two cannabinoid receptors, namely CB1R and CB2R [29]. Besides, there are other CBRs found to produce a protective role in CNS, including α7-nicotinic acetylcholine receptor [30] and CB52 receptor [26]. So far, CB1R has been proved to play a fundamental role in neuroprotection, which is mainly located in CNS [31]. However, CB2R is mainly found in periphery, especially in the immune system, majorly functioning in macrophage, microglia, and other immune cells, acting as immunomodulator [27]. There is already evidence that CB1R has fundamental neuroprotective roles in MS or EAE [29]. However, several works have been reported to show that selective activation of CB2R help ameliorate the initiation and development of MS or EAE, mainly through inflammary regulation and immunomodulation [24,32]. Here, we select CB2R as the researching target, because it is

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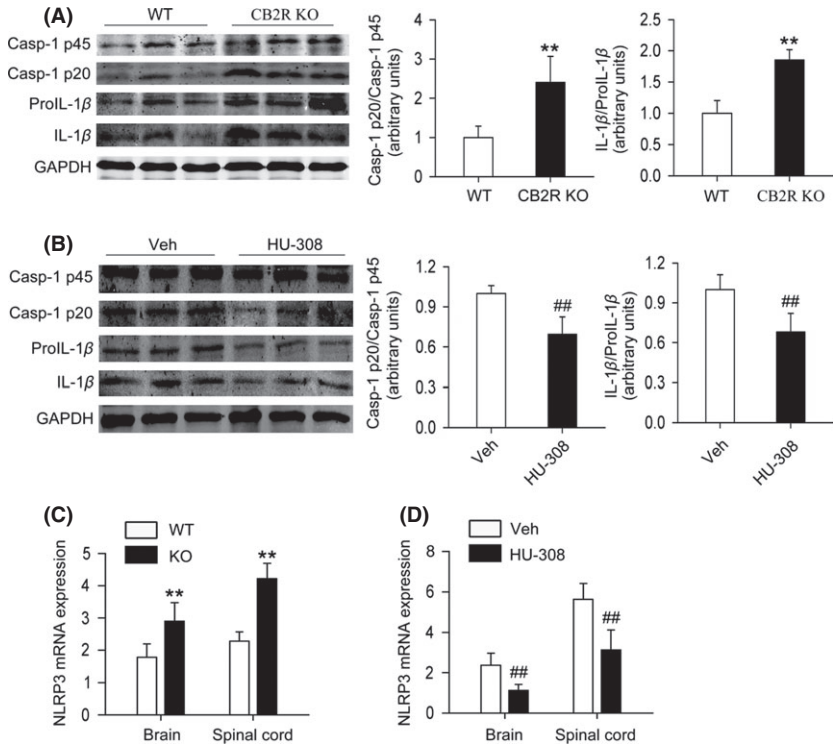

Figure 2 CB2R mediates inflammasome activation in EAE mice. (**A**–**B**) Spinal cords were isolated from EAE mice on day 10 PI and then lysed with buffer. Caspase 1 (Casp-1) activation and IL-1β production were analyzed using Western blotting. CB2R knock-out (KO) increased Casp-1 activation and IL-1β production, whereas activation of CB2R with HU-308 induced inverse effect (n = 6 per group). $^{**}P < 0.01$ versus wild-type (WT); $^{\#\#}P < 0.01$ versus Veh. (**C**–**D**) Spinal cords and brains were isolated from EAE mice, and levels of NLRP3 mRNA were analyzed with real-time PCR. CB2R-KO increased levels of NLRP3 mRNA in both spinal cord and brain, whereas activation of CB2R with HU-308 induced inverse effect (n = 6 per group). $^{**}P < 0.01$ versus WT, $^{\#\#}P < 0.01$ versus Veh.

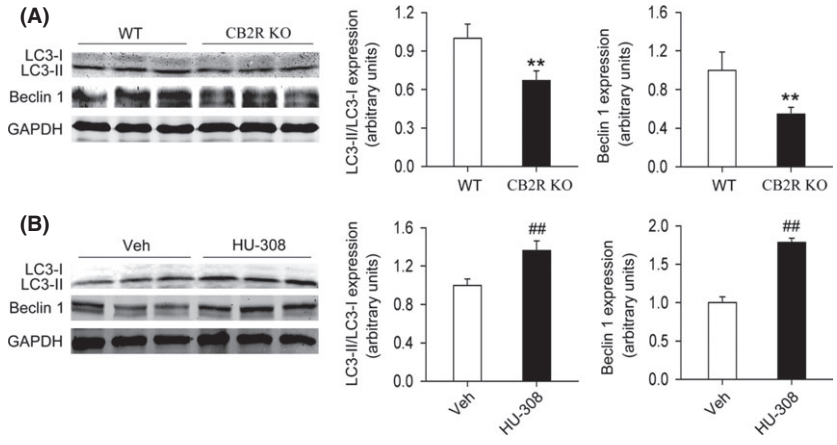

Figure 3 CB2R increases autophagy *in CNS*. Spinal cords were isolated from EAE mice on day 10 PI, and then lysed with buffer. (**A**) CB2R-KO decreased the expression of LC3-II/LC3-I ratio and Beclin 1 (n = 6 per group). $^{**}P < 0.01$ versus WT. (**B**) Activation of CB2R with HU-308 significantly increase the expression of LC3-II/LC3-I ratio and Beclin 1 (n = 6 per group). $^{\#\#}P < 0.01$ versus Veh.

believed that inflammation process is critical in the initiation and development of MS or EAE, which is mainly caused by migration and infiltration of inflammatory cells in CNS [33]. However, CB1R is mainly related to basic neuroprotection, seldom connected with inflammatory modulation [27]. In addition, established evidence has indicated that CB1R disturbed cholesterol metabolism and enhanced the risk of obesity and atherosclerosis [34]. In contrast, selective CB2R agonists show a positive and protective effect on metabolism and cardiovascular system [34]. In our study, we proved for the first time that a small dose of HU-308 (1 mg/Kg), a CB2R agonist, contributes greatly to the reduction of peak severity and cumulative clinical score of EAE mouse. It also led to less extensive demyelination and leukocyte infiltration in spinal cord. These results indicate that activating CB2R significantly alleviates EAE severity and downregulate inflammatory reaction and demyelination in CNS.

In addition, we found that deletion of CB2R increased the activation and expression of NLRP3e and inflammasome-related IL-1β and Casp-1, while activating CB2R with HU-308 decreased them. Further, we measured the changes of autophagy-related proteins such as LC3 and Beclin 1. The results showed the same trend as those of NLRP3, IL-1β, and Casp-1 along with the intervening regulation of CB2R. Then using ATG5-specific siRNA to interfere autophagy in BV2 microglia, we for the first time drew the conclusion that autophagy regulate CB2R-mediated NLRP3

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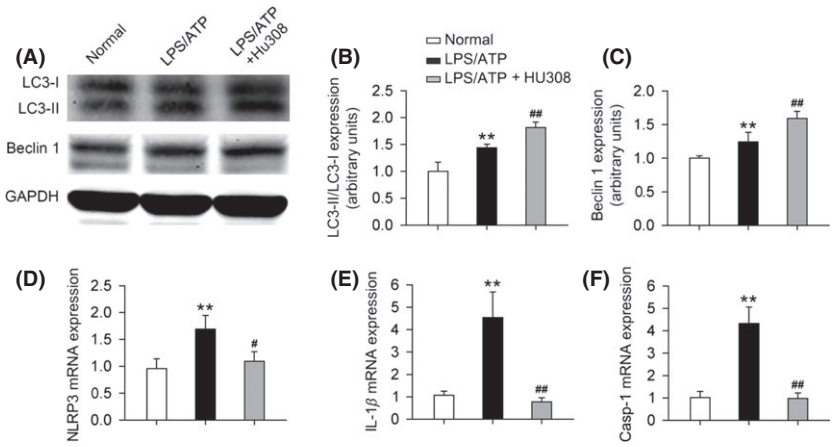

Figure 4 CB2R agonist HU-308 induces autophagy and inhibits NLRP3 inflammasome activity in BV2 microglia. BV2 microglia were pretreated with vehicle or HU-308 (10 μM) for 10 min and then stimulated with LPS (100 ng/mL) and ATP (1 mM). (**A**–**C**) BV2 microglia were lyzed and expression of LC3-II/LC3-I ratio and Beclin 1 were analyzed using Western blotting. HU-308 significantly increase the expression of LC3-II/LC3-I ratio and Beclin 1 (n = 6 per group). $^{**}P < 0.01$ versus Normal; $^{\#\#}P < 0.01$ versus LPS/ATP. (**D**–**F**) Cells were housed and mRNA levels of NLRP3, Casp-1 and IL-1β were analyzed with real-time PCR. HU-308 significantly decrease the mRNA levels of NLRP3, Casp-1, and IL-1β (n = 6 per group). $^{**}P < 0.01$ versus Normal; $^{\#}P < 0.05$ versus LPS/ATP; $^{\#\#}P < 0.01$ versus LPS/ATP.

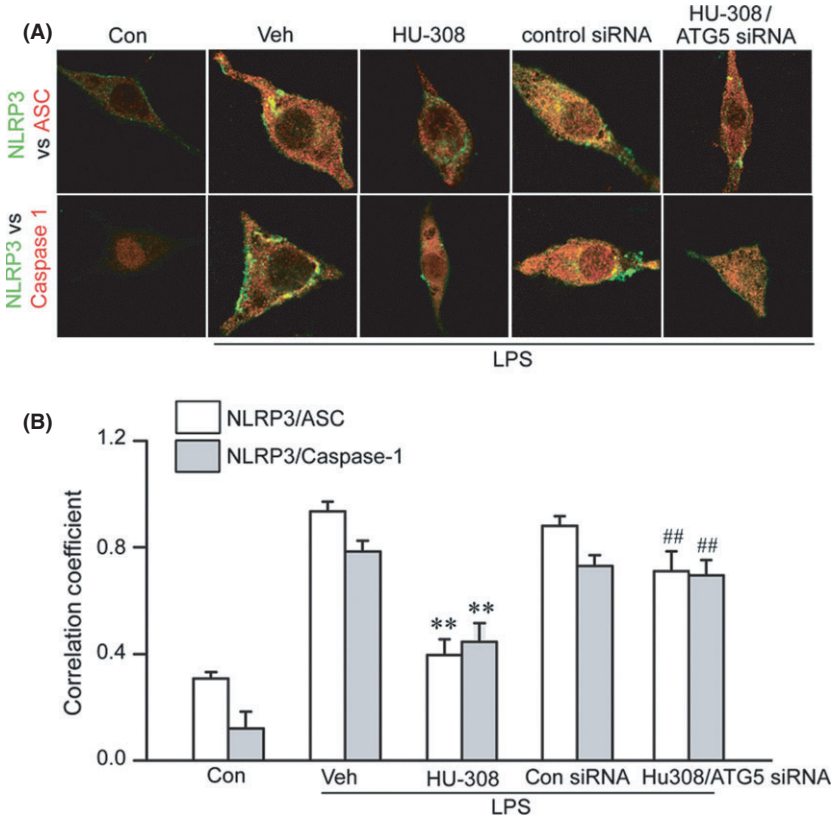

Figure 5 Blocking autophagy by ATG5 siRNA inhibits the effect of CB2R on NLRP3 inflammasome formation *in vitro*. (**A**) Confocal images representing the colocalization of NLRP3 (green) with ASC or caspase-1 (red) in cultured BV2 microglia. (**B**) Quantitative analysis showing the fold change in Pearson coefficient correlation (PCC) for the colocalization of NLRP3 with ASC and NLRP3 with caspase-1 (n = 6 per group). $^{**}P < 0.01$ versus control, $^{\#\#}P < 0.01$ versus veh, $^{\&\&}P < 0.01$ versus HU-308.

inhibition and at least partly mediate the inhibitory effect of CB2 agonists on NLRP3 inflammasome formation and activation in microglia. Generally, autophagy is thought to be a self-protecting cellular catabolic pathway relying on lysosomes [35]. Some long-lived proteins, as well as damaged organelles and misfolding proteins are degraded and recycled through autophagy process [35]. It is widely considered that autophagy acts as a downregulatory factor in inflammatory responses and inhibits the formation and activation of NLRP3 inflammasome as well [36,37]. A series of studies demonstrate that autophagy, especially in microglia, contributes greatly to ameliorating MS or EAE, because of its traits of antiinflammation and clearance of damaged organelles and

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protein aggregation, which lead to reduced inflammatory reaction, apoptosis, and necrosis [38]. However, recently, Bhattacharya et al. [39] showed that conditional inhibition of autophagy in dendritic cells (DC) alleviated demyelination and inflammatory infiltration in EAE mouse. We consider that the difference in target cell may lead to the inconsistency of the ultimate conclusion.

Autophagy is considered to promote the function of antigen-presenting cells (APCs) in immune and inflammation responses [40], and the overactivation of APCs results in the enhancing infiltration of inflammatory cells and the increasing intensity of immunoinflammatory reaction [39]. However, we focus on the effects of autophagy on microglia and demonstrated that activating the inflammation-related CB2R in microglia could ameliorate EAE through the induction of autophagy and inhibition of NLRP3 inflammasome initiation and activation. To ultimately take advantage of autophagy in treating MS, further researches and works are warranted.

Here, we used ATG5 siRNA to inhibit autophagic process. Generally speaking, ATG5 is one of the most critical autophagy-related genes, participating in the formation of autophagosome, the major functional unit of autophagy [40]. The method of ATG5 knockdown is selected by a series of works for the aim of autophagy inhibition [41]. However, many other autophagy inhibitors are found and used in research. For example, 3-methyladenine (3-MA) is found to inhibit the formation of autophagosome [42]. Nevertheless, as it inhibits all classes of PtdIns3K and the following signaling cascades, its specificity in inhibiting autophagy ought to be taken into account carefully [42]. Another one is chloroquine (CQ), which blocks the lysosomal proteolysis and lysosomal degradation, hence inhibiting the autophagic efflux [43]. However, when used to block autophagy, it may lead to the increase of autophagosome and the enhancement of autophagy-related proteins, which may be difficult for researchers to determine the inhibition of autophagy [43]. Thus, we think that data obtained from the last cell experiment would be more accurate and convictive if other kinds of autophagy inhibitors could be used in BV2 microglia, which might bring us the information of the influences of different autophagy phases in HU-308 and its antiinflammasome effect.

In summary, we demonstrate that activating CB2R significantly alleviates the pathogenesis and severity of EAE through the activation of autophagy and inhibition of NLRP3 inflammasome. This action of CB2R presents a novel insight into the molecular and signaling mechanism of its role on MS or EAE, which provides a good approach in study and treatment of MS. However, there are still quite a lot of questions to be answered and the specific mechanisms of MS still leave unknown. Thus, more detailed researches and statistical analysis are demanded in the battle against MS.

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Conflict of Interest

The authors declare no competing financial interests.

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Supporting Information

The following supplementary material is available for this article:

Table S1. Real-time PCR primers: the sequences of the primers used in QPCR were listed as below.

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